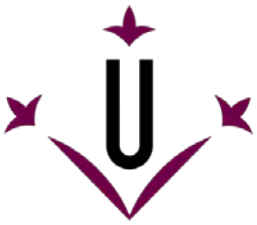

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Cross-Species Infectivity of H3N8 Influenza Virus in an Experimental Infection in Swine

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ABSTRACT

Avian influenza A viruses have gained increasing attention due to their ability to cross the species barrier and cause severe disease in humans and other mammal species as pigs. H3 and particularly H3N8 viruses, are highly adaptive since they are found in multiple avian and mammal hosts. H3N8 viruses have not been isolated yet from humans; however, a recent report showed that equine influenza A viruses (IAVs) can be isolated from pigs, although an established infection has not been observed thus far in this host. To gain insight into the possibility of H3N8 avian IAVs to cross the species barrier into pigs, *in vitro* experiments and an experimental infection in pigs with four H3N8 viruses from different origins (equine, canine, avian, and seal) were performed. As a positive control, an H3N2 swine influenza virus A was used. Although equine and canine viruses hardly replicated in the respiratory systems of pigs, avian and seal viruses replicated substantially and caused detectable lesions in inoculated pigs without previous adaptation. Interestingly, antibodies against hemagglutinin could not be detected after infection by hemagglutination inhibition (HAI) test with avian and seal viruses. This phenomenon was observed not only in pigs but also in mice immunized with the same virus strains. Our data indicated that H3N8 IAVs from wild aquatic birds have the potential to cross the species barrier and establish successful infections in pigs that might spread unnoticed using the HAI test as diagnostic tool.

IMPORTANCE

Although natural infection of humans with an avian H3N8 influenza A virus has not yet been reported, this influenza A virus subtype has already crossed the species barrier. Therefore, we have examined the potential of H3N8 from canine, equine, avian, and seal origin to productively infect pigs. Our results demonstrated that avian and seal viruses replicated substantially and caused detectable lesions in inoculated pigs without previous adaptation. Surprisingly, we could not detect specific antibodies against hemagglutinin in any H3N8-infected pigs. Therefore, special attention should be focused toward viruses of the H3N8 subtype since they could behave as stealth viruses in pigs.

The most ubiquitous hemagglutinin (HA) subtype of influenza A virus (IAV) is the H3, since it can be found in a variety of AQ:B organisms, including humans, pigs, horses, dogs, cats, seals, poultry, and wild aquatic birds. Among all H3 subtypes, the H3N8 has turned out particularly interesting since it has established lineages not only in wild aquatic birds but also in mammalian species such as horses and dogs. At present, H3N8 is the only IAV subtype circulating in equine and canine species (1, 2). However, until now this subtype is not circulating in pigs and humans (1).

Recently, equine H3N8 (clade II) strains have been isolated from pigs in China (3), but except for this publication no more data about transmission of equine IAV to pigs has been reported. Equine IAV has not been reported to cause disease in humans; however, a study from the 1960s showed that, in fact, humans were susceptible to equine IAV infection when infected with A/Equine/Miami/1/63 (H3N8) (4). Also, studies with archeoserological samples suggested that the virus circulating in humans during the 1889 pandemic could belong to the H3N8 subtype (5). Nevertheless, recent reports showed on one hand, sparse seroconversion in humans after exposure to equine IAV (6), and on the other hand, serological evidence of equine IAV infections among persons with horse exposure (7).

Seals on the coast of Massachusetts were recently dying from respiratory pneumonia, and the agent responsible for this deadly event was identified as an H3N8 virus of avian origin (8). The seal IAV was closely related to an avian strain, A/blue-winged teal/Ohio/926/2002 (H3N8), with an overall 96.07% nucleotide identity. This H3N8 seal IAV naturally acquired mutations known to

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increase transmissibility and, subsequently, ferrets were successfully infected by respiratory transmission (9).

Since the species barrier for H3N8 IAV might be easily overcome in nature, we hypothesized that H3N8 viruses from different origins had acquired the ability to replicate and produce lesions/disease in pigs. Thus, four H3N8 IAVs from different species were selected to assess the ability to replicate and produce disease in pigs.

Our results showed that swine, avian, and seal IAVs exhibited higher replication abilities and differential binding affinities *in vitro* than the equine and canine IAVs. Moreover, H3N8 viruses adapted to equine and canine exhibited negligible replication in inoculated pigs, and therefore no lesions were found in these animals. Surprisingly, the H3N8 avian virus selected in the present study, as well as the virus isolated from seals not only replicated substantially but also caused lesions in the lungs of infected pigs. Interestingly, we were unable to detect antibodies by hemagglutination inhibition (HAI) test in animals when infected with the avian or the seal H3N8 viruses. The absence of antibodies as determined by HAI was also observed in mice immunized with the same avian and seal viruses.

MATERIALS AND METHODS

Virus. The following viral isolates were used for the *in vitro* experiments and the experimental infection: A/Swine/Spain/54008/2004 (H3N2), a swine IAV strain (Sw); A/Equine/OH/1/03 (H3N8), an equine IAV strain (Eq); A/Canine/NY/105447/08 (H3N8), a canine IAV strain (Ca); A/American black duck/Maine/44411-532/2008 (H3N8) (Av); and A/Harbor Seal/New Hampshire/179629/2011 (H3N8) (Se). Also, a low-pathogenicity A/Anas plathyrhynchos/Spain/1877/2009 (H7N2) strain, a pandemic A/Catalonia/63/2009 (H1N1) strain, and a human A/Perth/16/09 (H3N2) strain were used. Viral stocks were grown in 10-day-old specific-pathogen-free (SPF) embryonated eggs or in MDCK as stated in the text.

Mean death time and viral growth. Ten-day-old embryonated eggs were inoculated with 0.1 ml of Sw, Eq, Ca, Av, and Se viruses containing 10^4 and 10^5 50% chicken embryo infectious doses (EID_{50}), respectively. Three eggs per sample were used. Eggs were candled every 6 h to determine death over 4 and a half days. The time in hours needed to kill half of the inoculated embryos for each virus dilution was then calculated. The mean time to death (MTD) in hours represents the average of three values.

To analyze viral replication, confluent MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 and incubated for different time periods at 37°C. Culture supernatants were collected at different time points, and progeny viruses were determined by plaque assay. Titers were expressed as the number of PFU per ml.

Hemagglutination assays. Hemagglutination assays were performed for pigs according to the OIE manual for testing terrestrial animals. Briefly, red blood cells (RBCs) from chicken, horse, cow, sheep, ferret, pig, dog, guinea pig, and goat sources were obtained from the experimental farm at the Universitat Autonoma de Barcelona (Spain) and kept in Alsever's solution (Sigma). RBCs from humans were obtained from healthy volunteers. The RBCs were washed in phosphate-buffered saline (PBS) and resuspended to 0.5% (chicken) or 0.75% (other species). Viruses were diluted to 4 HA units/50 μl using chicken RBCs. Serial dilutions of test sera were then prepared, added to equal volumes of washed RBCs of each species, and incubated in V-bottom plates. Reaction mixtures were incubated at room temperature for 30 min. The HA titer endpoint was the reciprocal of the highest dilution that causes complete hemagglutination.

Influenza A virus binding to swine tracheal explants. The tracheas from two mock-infected pigs were obtained under aseptic conditions. After the samples were washed with PBS supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml), fragments of about 5 by 5 mm^2 were excised and kept on ice until use. Control tracheal explants devoid of sialic acid residues were prepared by incubation with neuraminidase (NA; Sigma catalog no. N7885, 10 U/ml) for 2 h at 37°C. Host receptor binding assays with Sw H3N2, Eq H3N8, and Ca H3N8 were performed as follows. First, 10^3 EID_{50} of each virus were added to four explants (two from each of two tracheas) in each well of a six-well plate with 1 ml of culture medium (Dulbecco modified Eagle medium [DMEM] and RPMI 1640 [1:1]), along with penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml), followed by incubation for 1 h at room temperature. Neuraminidase-treated control tracheal explants were also inoculated in parallel. After incubation, the virus was removed, and the explants were washed 10 times with PBS with antibiotics and frozen at −80°C. The total RNA from the explants was isolated with a PureLink RNA minikit (Ambion, catalog no. 12183018A), and an IAV M-gene-based real-time reverse transcription-PCR (~~r~~RT-PCR) was performed in accordance with CDC ~~r~~RT-PCR protocols.

Detection of swine influenza in swine tracheal explants. IAV attached to tracheal explants was detected as previously described (10) by using a Superscript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, catalog no. 11732-088), including a 25-μl reaction, run on a Corbett Research Rotor-Gene 3000 real-time thermal cycler. The cycle threshold (C_T) values for the rRT-PCR of the different explant and virus samples were recorded to assess the relative amounts of virus bound to the tracheal explants that were wither treated and not treated with neuraminidase.

IAV proliferation in tracheal explants was detected by infecting two six-well plates, each containing six tracheal explants, with 10^6 EID_{50} of each strain in 1 ml of infection medium (DMEM-RPMI [1:1]), including penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (100 μg/ml), and then treated with trypsin (2 μg/ml) for 1 h at 37°C. After infection, explants were washed six times with 2 ml of PBS, followed by incubation in 1 ml of infection medium at 37°C. Samples of 150 μl of supernatant were taken at 0, 24, and 48 h postinfection (p.i.) and frozen at −80°C until analysis. Quantification of virus in supernatants was performed by using rRT-PCR as described above and according to the method of Reed and Muench (11). At each time point after infection, two explants were taken and fixed with 10% buffered formaldehyde for immunohistochemistry analysis.

Animals. All experiments with pigs were conducted at the Centre de Recerca en Sanitat Animal, Barcelona, Spain, in compliance with the Ethical Committee for Animal Experimentation of the institution (Universitat Autònoma de Barcelona). The treatment, housing, and husbandry conditions conformed to European Union guidelines (Directive 2010/63/EU on the protection of animals used for scientific purposes). Animal care and procedures were in accordance with the guidelines of the *Good Laboratory Practices* under the supervision of the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona (approval no. 1189) and under the supervision of the Ethical and Animal Welfare Committee of the Government of Catalonia (approval no. 5796). In line with the above operational framework, six groups of pigs (7 to 8 weeks old, Landrace × Pietrain, free from common pathogens) were housed in separate isolation rooms and adapted to the new environment and stockmen over a 1-week period under veterinary supervision. Experiments with mice (BALB/c strain, Harlan Laboratories Models, Spain) were conducted at the INIA (Instituto Nacional de Investigación y ~~Tecnologša~~ Agraria y Alimentaria) facilities in Madrid, Spain, in compliance with the Ethical Committee for Animal Experimentation guidelines of the institution.

Experimental infection. The pigs used in our experiment were seronegative to influenza A viruses by competition Elisa (ID Screen Influenza A Antibody Competition ELISA; ID-Vet, France) at the time of the experiment. Five groups of 12 pigs were infected with IAV strains Sw H3N2, Eq H3N8, Ca H3N8, Av H3N8, and Se H3N8, respectively. Six pigs were mock infected.

On day 0, each pig in all groups but the mock-treated group was

intratracheally infected with 1 ml of virus containing 2×10^5 EID_{50} of the corresponding virus strain. Animals were clinically inspected, and the rectal temperature was recorded on a daily basis. Four pigs of each virus-infected group were euthanized and weighed at 3, 6, and 21 days p.i., respectively. In the mock-infected group, two pigs were euthanized and weighed at each of the same times. For all of the animals, the average daily weight gain was recorded as the difference between the final and initial weights (day 0 p.i.) divided by the number of days postinfection (dpi).

Samplings and postmortem examination. Nasal swabs were taken at days 1, 2, 4, 8, and 10 p.i. They were frozen for viral determination. Venous blood samples in vacuum tubes were collected at day 0 and just before sacrifice on the aforementioned days. After sacrifice, bronchoalveolar fluids (BALF) were collected via lung lavage with PBS, as described by Busquets et al. (12). Briefly, the right lungs of sacrificed pigs was used to perform a bronchoalveolar lavage (BAL) using approximately 100 ml of PBS, and the left one was sampled for histopathological and virological studies. A complete necropsy was performed. Lung lesions were classified depending on the extension of pneumonia. Mild lesions were recorded when affecting small areas (<2 cm^2) of cranial or medial lung lobes, moderate lesions were recorded when affecting extended areas (2 to 5 cm^2) of cranial or medial lobes, and severe lesions were recorded when affecting large areas (>5 cm^2) of cranial, medial, diaphragmatic, and accessory lobes.

Histopathology and immunohistochemistry. Samples from trachea and lungs were collected, fixed in 10% buffered formalin, and processed for histopathology, i.e., they were dehydrated through graded alcohols and embedded in paraffin. Sections (3 μm thick) were cut, stained with hematoxylin-eosin, and examined in a "blind fashion." In particular, bronchiolar epithelial changes and peribronchiolar inflammation was evaluated in large, medium, and small or terminal bronchioles, as well as inflammatory changes in alveoli. In the lung, bronchointerstitial pneumonia intensity was assessed by means of semiquantitative lesion scoring. The pathological scores for tracheal and pulmonary tissues were as follows, depending on the extension of the inflammation: 0, no lesions; 1, mild lesions; 2, moderate lesions; and 3, severe lesions. Immunohistochemistry was used to detect influenza A virus nucleoprotein (NP) according to previously described methods (13).

IAV titration. Nasal swabs were collected and tested to the determine virus load as described by Busquets et al. (12). Lung homogenates were prepared by grinding the tissue; homogenates were suspended in 1 ml of PBS, and tissue debris was removed by low-speed centrifugation. Virus titers were determined by serial 10-fold dilutions in PBS and inoculation into the allantoic cavities of five 10-day-old SPF chicken embryos. Samples were tested for influenza A virus by using a real-time RT-PCR (14) for influenza A matrix gene.

Antibody assays. Serum samples collected from pigs killed at days 3, 6, and 21 were tested for antibodies against the NP of influenza A virus (ID Screen Influenza A Antibody Competition ELISA; ID-Vet, France). BALF and serum collected from pigs killed at days 3, 6, and 21 were tested for antibodies against NP of influenza A virus using the same ELISA. 1/10 and 1/20 dilutions of each sample were examined according to the method of de Boer et al. (15). Samples were considered positive if at least 75% inhibition was detected at either dilution.

The HAI test was performed on pig sera collected at days 0, 3, 6, and 21 against the respective homologous IAV strains according to the directions of the *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (16). Heterologous swine and equine IAV were also used in HAI tests (swine IAVs H1N1 A/sw/It/267505, H1N2 A/sw/It/284922/09, and H3N2 A/sw/It/312583/09 and equine IAV H3N8 A/eq/New Market/93).

A neuraminidase inhibition assay was performed according to recommended World Health Organization protocols (17) and is based on the ability of antisera to inhibit the activity of the NA enzyme of IAV determined by the release of free sialic acid from a fetuin substrate (18). The amount of free sialic acid was measured in a spectrophotometer at 549 nm, and one unit of NA activity was determined as the dilution of virus that gave an optical density reading of 0.500 under standard conditions. Serum dilutions from pigs infected with each indicated virus, collected 21 days p.i., were preincubated for 60 min at room temperature with one unit of NA activity of the corresponding virus. The percentage of NA activity not neutralized by antiserum, with respect to the negative control, was plotted against an antiserum dilution.

TABLE 1 Mean time to death[a]

Influenza virus	Mean time to death (h) ± SD	
	10^4 EID_{50}	10^5 EID_{50}
Sw	50 ± 7.5	50 ± 2.8
Eq	>108	>108
~~Ca~~	>108	>108
Av	60 ± 4.9	58 ± 5.6
Se	96 ± 8.5	96 ± 4.9

[a] Three ten-day-embryonated chicken eggs were inoculated with 10^4 and 10^5 50% egg infective doses (EID_{50}) of swine (Sw), equine (Eq), canine (Ca), avian (Av), or seal (Se) influenza viruses. Eggs were candled every 6 h to determine death over 4.5 days.

A virus neutralization assay was performed according to recommended World Health Organization protocols (17) with the following modifications. Briefly, 50 μl of a stock solution (2,000 50% tissue culture infective doses [$TCID_{50}$]/ml) of virus was mixed with 50 μl of 2-fold dilutions of sera. After 2 h of incubation at 37°C, virus-serum mixtures were added to 96-well microtiter plate containing MDCK cells (120,000/0.050 ml/well). To detect viral infection, each well was observed at microscope after 80% acetone fixation for 48 h at −20°C and staining with a nucleoprotein IAV monoclonal antibody (ATCC hb65) conjugate with peroxidase. Titers for the SN assays were reported as the reciprocal of the last serum dilution that completely inhibited infection of MDCK cells.

Immunization of mice. BALB/c female, 8-week-old mice (four mice per group) were inoculated intraperitoneally on days 0 and 14 p.i. with 2.7×10^7 EID_{50} or 6 HA units per mouse of Sw H3N2, Av H3N8, and Se H3N8 IAVs inactivated by UV radiation. For UV inactivation, 0.5 ml of the virus stocks were placed in a 12-well plate and exposed to 4,000 ergs/cm^2 from a UV source for 15 min at 4°C. Inactivation of viruses was confirmed by the inoculation of three 10-day-old embryonated chicken eggs with 100 μl of each UV-treated stock virus. A control group of non-immunized mice was used for comparison.

Blood samples were collected 2 weeks after the second immunization, and sera previously treated at 56°C for 1 h and with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO) at 37°C overnight were analyzed by enzyme-linked immunosorbent assay (ELISA) and HAI testing. Titers of anti-influenza A virus antibodies of mice sera were tested in a homemade ELISA using microplates coated with 2 μg of purified A/PR/8/34-H1N1 strain/ml. HAI titers were assayed as previously described for swine sera and according to established protocols (16).

Viral genome sequence. Viral RNA was extracted from samples by using a QIAamp viral RNA minikit according to the manufacturer's instructions (Qiagen). RT-PCR analyses of all eight genome segments was performed as described by Lycett et al. (19) using the SuperScript III One-Step RT-PCR system with Platinum High Fidelity *Taq* (Invitrogen). RT-PCR products were purified with NucleoSpin gel and PCR Cleanup (Macherey-Nagel). DNA was quantified using a Sinergy H1 hybrid reader (Bio-Tek). Indices were added, and multiplexed DNA libraries were made with a Nextera-XT kit (Illumina) according to the manufacturer's instructions. Pooled libraries were sequenced on a MiSeq Instrument (Illumina) by using a Miseq reagent kit (v2) in a 250-cycle paired-end run. The data were *de novo* assembled on cloud on Base Space (BaseSpace; Illumina) by the DNASTAR application and analyzed by the Lasergene Package software (v10.1.2).

Full genome sequences obtained or retrieved from GenBank were aligned by CLUSTAL W using MEGA5, and trees were produced using the neighbor-joining method with a 1,000-replicate bootstrap. The evolu-

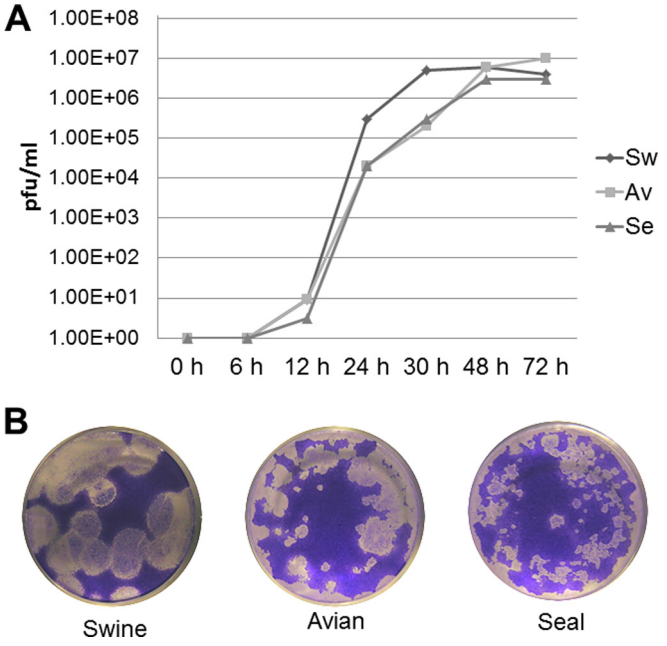

FIG 1 (A) MDCK viral growth *in vitro* of swine (Sw), avian (Av), and seal (Se) IAVs. (B) Plaque sizes of Sw, Av, and Se IAVs on MDCK cells.

tionary distances were computed using the Kimura two-parameter method with bootstrap test (1,000 replicates). Sequences of eight gene segments of the seal, avian, and swine viruses, before and after the replication in pigs were aligned, respectively, by CLUSTAL W using MEGA5 (20). The predicted amino acid sequence was obtained for each gene, and a comparison between the proteins before and after the infection was performed.

Statistical analysis. All statistical analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL). For all analyses, each animal (pig or mouse) was used as the experimental unit. The significance level (α) was set at 0.05, with statistical tendencies reported when P was <0.10. A nonparametric test (Kruskal-Wallis) was chosen to compare the values obtained for the viral load, antibody titer, body temperature, and average daily gain between the different viruses used for the *in vivo* experiment. This same statistical tool was also used to compare the viral load determined for the *in vitro* experiment for the different groups. Finally, a Fisher exact test was used to test the association between histopathological scores, macroscopic lesions, and the different viruses used for the *in vivo* experiment, respectively.

RESULTS

Swine, avian, and seal IAVs exhibited higher replication abilities *in vitro* than the equine and canine IAVs. First, replication of the different H3N8 IAVs was analyzed *in vitro*. Replication in different substrates (embryonated chicken eggs and MDCK cells) was performed successfully by all viral strains except the canine IAV. The canine strain was not able to grow in MDCK cells, although different cell batches were tested. Also, the equine strain grew very poorly. Since all viruses replicated in eggs, they were grown in 10-day-embryonated eggs, and titers were determined by EID_{50} to standardize the virus stocks. The obtained virus titers were: swine (Sw), $EID_{50}/ml = 5.6 \times 10^7$; equine (Eq), $EID_{50}/ml = 5.6 \times 10^7$; canine (Ca), $EID_{50}/ml = 3.2 \times 10^7$; avian (Av), $EID_{50}/ml = 3.2 \times 10^8$; and seal (Se), $EID_{50}/ml = 5.6 \times 10^8$. In order to compare the abilities of these viruses to kill embryonated eggs, 9-day-embryonated eggs were inoculated with two different doses (10^4 and 10^5 EID_{50}, respectively), and the mean time to death (MTD) for each viral strain was measured (Table 1). Although the Eq and Ca viruses were not able to kill the eggs in 108 h, the MTD for Sw IAV was ~50 h, that for Av IAV was 58 to 60 h, and that for Se IAV was ~96 h.

Subsequently, the growth of the Av and Se H3N8 viruses was compared to that of Sw H3N2 virus on MDCK cells infected at an MOI of 0.001 (Fig. 1A). The Eq and Ca viruses were not included, since replication in MDCK cells was undetectable. All tested viruses grew in MDCK cells reaching similar titers (Fig. 1A), although the Sw IAV showed a faster replication in this substrate. Then, 150-μl portions were taken at each time point and used to assess the virus titers by a plaque assay. Remarkably, Sw IAV formed the largest plaques, followed by the Av IAV; Se IAV yielded the smallest plaques of the three viruses (Fig. 1B).

Equine, canine, avian, and seal IAVs exhibited different binding affinities to a broad repertoire of RBCs. IAV viruses bind to cell surface sialyloligosaccharides with specificities that vary according to the host species of origin. A rough method to determine receptor specificity of IAV is to measure the ability of these viruses to hemagglutinate different RBCs, as it has been previously shown (21). A panel of RBCs from different sources were

TABLE 2 Hemagglutination activity of Sw H3N2, Eq H3N8, Ca H3N8, Av H3N8, and Se H3N8 influenza viruses with erythrocytes from different animals[a]

	HA titer endpoint (~~μg/ml~~)								
Influenza virus	Horse	Cow	Sheep	Ferret	Human	Pig	Dog	Guinea pig	Goat
Sw H3N2	32	<2	4	4	16	4	16	16	2
Eq H3N8	32	32	16	16	16	4	32	16	8
Ca H3N8	32	32	16	16	16	8	32	16	4
Av H3N8	32	32	16	32	16	16	32	16	8
Se H3N8	32	64	32	16	16	32	16	16	16
H7N2(LP)	32	16	16	16	16	16	16	16	4
pdmH1N1	8	2	8	16	16	<2	16	16	2
H3N2	2	2	<2	32	16	<2	16	16	<2
MAAII	>256	<2	32	128	128	4	>256	128	2
SNA	16	<2	2	>256	>256	>256	64	>256	<2

[a] Red blood cells (RBCs) from horse, cow, sheep, ferret, human, pig, dog, guinea pig, and goat sources were washed in PBS and resuspended to 0.75%. Viruses were diluted to 4 HA units established on chicken RBCs. Serial 2-fold dilutions of viruses were then prepared, added to equal volumes of washed RBCs of each species, and incubated in V-bottom plates. Reaction mixtures were incubated at room temperature for 30 min. The HA titer endpoint is the reciprocal of the highest dilution that caused complete hemagglutination.

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TABLE 3 Binding of influenza viruses on swine tracheal explants[a]

Influenza virus	Mean $C_T \pm$ SD		ΔC_T
	Without neuraminidase	With neuraminidase	
Sw	37.14 ± 1.73	40.14 ± 1.01	3.00
Eq	39.75 ± 0.15	39.88 ± 0.3	0.13
Ca	41.01 ± 0.31	42.32 ± 0.65	1.31
Av	38.74 ± 0.51	41.44 ± 0.54	2.70
Se	38.62 ± 0.74	40.83 ± 094	2.21

[a] C_T, cycle threshold values of matrix-based RT-PCRs of neuraminidase-treated or nontreated swine tracheal explants incubated *in vitro* with different influenza viruses. The means of six infected explants examined for each virus are shown.

used to investigate the avidity of our H3N8 IAV, as well as of the Sw H3N2 IAV. As a control, two lectins with proven different receptor specificity were used. *Maackia amurensis* lectin II (MAAII) binds sialic acid in an α-2,3 linkage. On the other hand, *Sambucus nigra* lectin (SNA) prefers structures with α-2,6-linked sialic acid (22). Another two human influenza A viruses, A/Catalonia/63/2009 (H1N1) and A/Perth/16/09 (H3N2), were used for comparison with the viruses under study. In addition, we also tested an unrelated low-pathogenicity IAV from a different subtype, A/Anas plathyrhynchos/Spain/1877/2009 (H7N2; accession no. KP636483 to KP636490) (23) (Table 2). T2/AQ:N

Chicken RBCs were bound by all IAV tested (data not shown), and we standardized the assay to four HA units of virus using chicken blood (the limited HA activity was determined by the low titer of the Ca IAV). Horse and cow RBCs have been reported to have predominant expression of α-2,3-linked sialic acids, and therefore human IAVs do not bind, whereas avian viruses do. Our results clearly correlated with the previous reported observations since the human viruses bind with no or low avidity to horse and cow RBCs. Strikingly, whereas MAAII binds with stronger avidity to equine RBCs, its binding to cow RBCs was completely negative in contrast to H3N8 and H7N2 viruses. The binding avidity to guinea pig and human RBCs was indistinguishable among all viruses tested and, in fact, both lectins, although at a higher avidities, bound in a similar way. RBCs from pigs could be agglutinated by all H3N8 viruses but not by the human viruses tested. Our lectin control showed that SNA could bind with a high efficiency; however, our two human IAV could not bind. Although swine and human IAVs showed distinct avidities when using different RBCs, the H3N8 viruses, particularly the Av and Se viruses, could bind to a wide repertoire of RBCs with similar avidities, i.e., to a broader range of sialic acids (Table 2).

Equine, canine, avian, or seal IAVs exhibited different binding and replication abilities in pig tracheal explants. One of the steps that IAVs must overcome to infect the respiratory tract in the host is to bind and replicate in the tracheal epithelium. Thus, the ability of the four H3N8 and swine IAVs to bind and replicate in swine tracheal explants was addressed *in vitro* (Tables 3 and 4). T3-4 AQ: IAV binding to tracheal explants versus binding to explants devoid of sialic receptors by neuraminidase treatment gave us quantitative evidence of specific virus binding to its receptor. Sw IAV showed the strongest specific binding to swine tracheal explants, followed by the Av and Se IAVs. Eq and Ca IAVs, however, did not show specific binding to explants (Table 3). A statistical tendency ($P = 0.08$) was observed in the binding ability between all of the pairs of viruses included in the study with the exception of Av/Se and Sw/Se. The proliferation of Sw, Eq, Av, and Se IAVs was also measured on supernatants of swine explants by determining the $TCID_{50}$ and by quantitative RT-PCR (qRT-PCR) (Table 4). Swine IAV and, to a lesser extent, the Av and Se IAVs were able to replicate in swine tracheal explants. However, Eq and Ca IAVs did not exhibit any measurable replication.

Equine, canine, avian, or seal IAVs differ in their pathogenicity in pigs. In order to gain insight into the ability of these viruses to cross species barrier and establish an infection into a mammalian host, groups of 12 pigs were infected with each of the five H3 IAV plus six mock-infected animals. None of the infected pigs showed respiratory signs after infection (data not shown) with the exception of fever in some animals belonging to the Sw and Av infected groups from days 2 to 4 p.i. (Fig. 2). The highest percentage of animals with fever was observed at day 3 p.i., and this percentage was significantly different between the Sw infected group and the rest of viruses, with the exception of the avian group, where a statistical tendency was observed ($P = 0.08$). No significant differences were observed in the average daily weight gain (ADWG) between pigs infected with different viruses at the different time points, but the lowest ADWG was observed for the Sw infected group (data not shown). F2

At 3 days p.i., macroscopic lesions were not observed in the lungs of pigs infected with the equine and canine IAVs nor in the mock-infected group. Extended areas of lung consolidation affecting 50% of both cardiac lobes and a small portion of right diaphragmatic lobe could be observed in swine IAV-infected animals. Interestingly, infection with Av and Se IAVs, on the other hand, resulted in multifocal areas of lung consolidation affecting 25% of both cardiac lobes (Fig. 3). Moreover, the macroscopic and microscopic lesions observed for the different viruses were significantly different ($P < 0.05$) between them. At 6 dpi, a pattern of F3

TABLE 4 Virus proliferation on tracheal explants: C_T and log $TCID_{50}$ values

Influenza virus	Mean $C_T \pm$ SD[a]			Log $TCID_{50}$[b]			
	0 hpi	24 hpi	48 hpi	Inoculum	0 hpi	24 hpi	48 hpi
Sw	39.40 ± 1.21	28.20 ± 1.35	26.50 ± 1.82	2.75	0.00	3.25	4.25
Eq	39.84 ± 0.74	39.34 ± 1.07	40.04 ± 1.62	2.25	0.00	0.00	0.00
Ca	40.08 ± 0.86	39.59 ± 1.22	41.38 ± 1.93				
Av	39.12 ± 1.23	30.66 ± 1.85	28.20 ± 0.86	4.00	0.00	3.20	1.37
Se	40.02 ± 1.42	32.40 ± 1.53	31.08 ± 1.87	4.00	0.00	2.25	2.70

[a] C_T, cycle threshold values of matrix-based RT-PCR of supernatants of swine tracheal explants Means of supernatants from six infected explants for each virus are shown.
[b] The virus concentration in supernatants from infected tracheas was measured in MDCK cell cultures at different time points. Note that canine H3N8 IAV does not proliferate on MDCK cells.

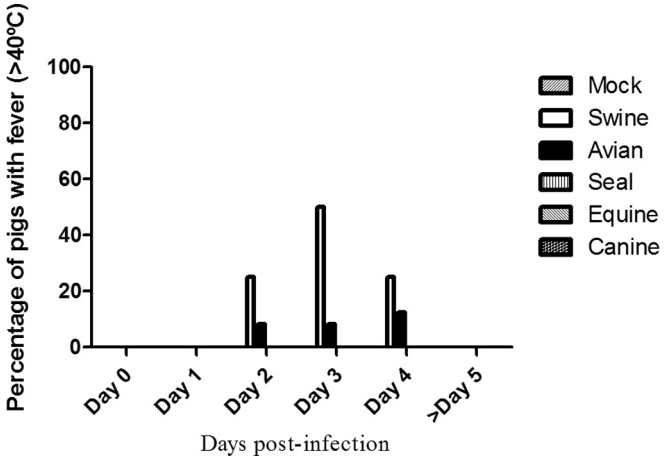


FIG 2 Percentage of animals with fever (>40°C) after IAV inoculation. Twelve pigs by group were inoculated with ~~swine-origin avian, seal, equine, and canine H3N8 IAVs~~, or twelve animals were mock infected or infected with H3N2 IAVs, respectively. The temperature was recorded daily. The data shown are averages from all animals per group.

AQ: Z

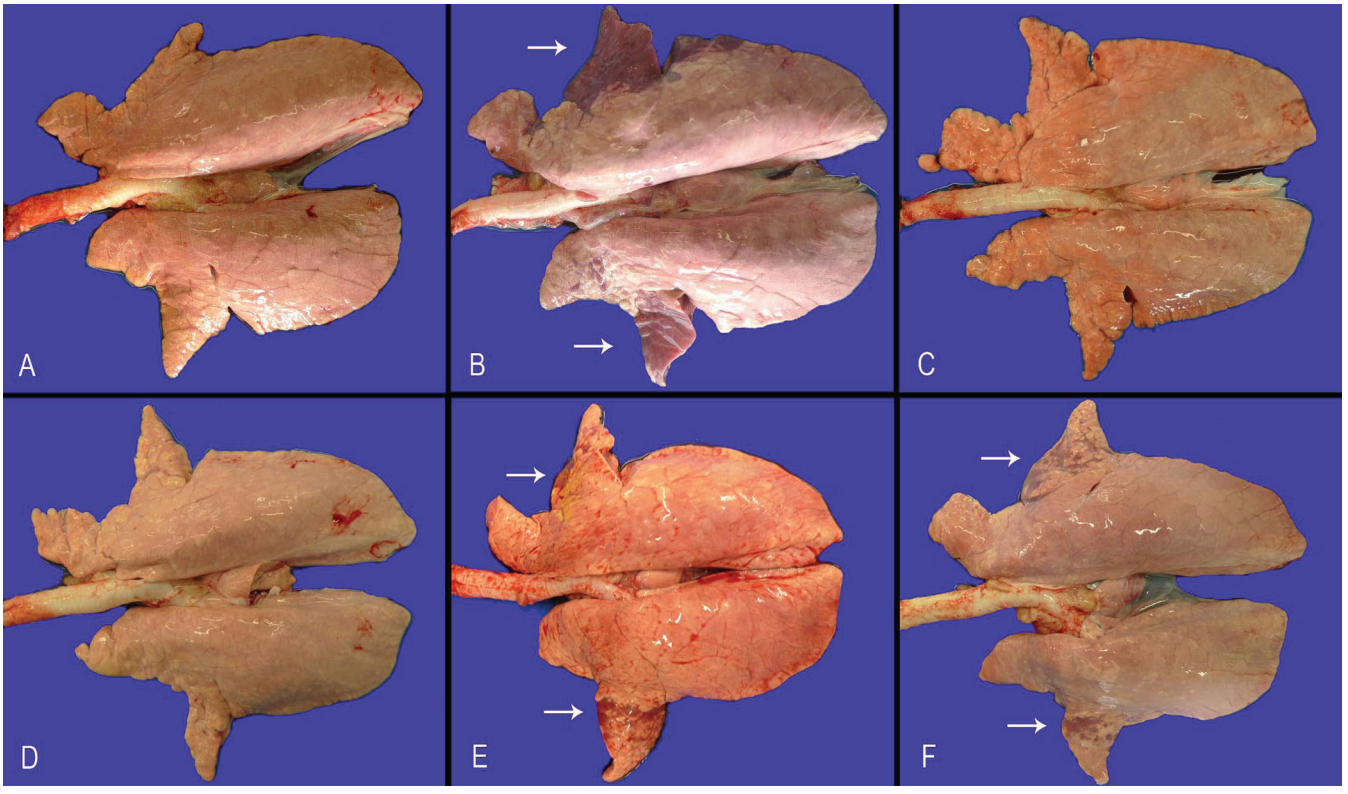
lung consolidation similar to the one observed at 3 dpi was observed in euthanized pigs. Finally, at 21 dpi, most pigs showed healed lungs, including the presence of scars in some cases (a finding reminiscent of a previous infection) (data not shown).

Histopathological and immunohistochemical studies further corroborated gross macroscopic lesions in the lungs (Table 5 and Fig. 4). Pulmonary lesions were consistent with bronchointerstitial pneumonia at different grades of severity. This lesion consisted of a lymphoplasmacytic infiltration in the bronchioli with extension to the surrounding alveolar septa. In the most severe cases, at 3 days p.i., necrosis of the bronchiolar epithelium was observed, with accumulation of necrotic cells in the bronchiolar lumen and alveolar spaces. Tracheal lesions consisted of variable grades of lymphoplasmacytic infiltration in the lamina propria; in the most severe cases, focal ulceration of the mucosa with fibrin deposition and neutrophilic infiltration was observed (data not shown). Immunohistochemical staining ~~of the IAV~~ was observed in the nuclei of tracheal and bronchiolar epithelial cells, pneumocytes, and alveolar macrophages. Positive staining was abundant in the most severe cases; however, only a few cells were positive in moderate lesions. No positive staining was detected in mild cases.

T5 F4 AQ: Q

Avian and seal IAV viruses replicated and maintained infections in the lungs of pigs without shedding. The ability of these IAV strains to replicate in lungs was investigated by analyzing EID_{50} virus titers in tissues. In parallel, using a different sample from the same infected area, the viral concentration was also determined by qRT-PCR (Table 6). The highest virus titers were obtained within the Sw virus-infected group at day 3 in the range of 10^6 to 10^7 EID_{50}/ml. Biological titers correlated with the qRT-PCR data. At day 6, two of four animals still had detectable EID_{50} virus titers in the range of 10^2 EID_{50}/ml, although viral RNA was also detected in samples at 6 dpi from all of the four pigs. Except

T6 Q:R



COLOR

FIG 3 Macroscopic pulmonary lesions observed at 3 dpi in pigs inoculated with different IAVs. (A) Control. Note the absence of lesions. (B) Swine (Sw) IAV. There are extended areas of pneumonia (arrows) affecting 50% of both cardiac lobes and a small portion of the right diaphragmatic lobe. (C) Equine (Eq) IAV. Note the absence of lesions. (D) Canine (Ca) IAV. Again, note the absence of lesions. (E) Avian (Av) IAV. There are multifocal areas of pneumonia affecting 25% of both cardiac lobes (arrows). (F) Seal (Se) IAV. There are multifocal areas of pneumonia affecting 25% of both cardiac lobes (arrows).

TABLE 5 Pathological and immunohistochemical analyses of tracheal and lung tissue from controls and pigs infected with different influenza viruses[a]

Time point (days)	Score																					
	Control				Swine				Equine				Canine				Avian				Seal			
	T		L		T		L		T		L		T		L		T		L		T		L	
3	0	–	0	–	1.5	+	2.25	+	0.25	–	0.5	–	0.25	–	0	–	1.25	–	2	+	1	–	1.75	+
6	NP	NP	NP	NP	1.5	NP	0.25	NP	NP	NP	NP	NP	NP	NP	NP	NP	1.25	NP	0.5	NP	1.5	NP	0.5	NP
21	NP	NP	NP	NP	0	–	0	–	NP	NP	NP	NP	NP	NP	NP	NP	0.75	–	0.75	–	0.25	–	1.25	–

[a] For each tissue, the pathological score varied from 0 to 3; the data represent the averages for each group (four animals), except for the controls (two animals). For immunohistochemistry, the detection of influenza antigen in the group is indicated (+/–). T, trachea; L, lung; NP, not performed.

for one animal in the Eq and Ca virus-infected groups with an extremely low virus titer, the presence of viral replication in the lungs of pigs infected with these viruses was undetectable. These results correlated with the absence of lesions in the lungs (Fig. 3) and the data from binding and replication in trachea explants (Tables 3 and 4). The Se IAV replicated to a certain extent in the lungs of pigs, between 10^2 and 10^3 EID_{50}/ml at day 3 p.i. Significant differences ($P < 0.05$) were observed in the ability of these viruses to replicate in the lungs between all pairs of viruses with the exception of the Sw/Av and Sw/Se IAVs. At day 6, the presence of the virus in one animal as determined by EID_{50} and in two animals by as determined by qRT-PCR was still detectable. Finally, the Av IAV replicated substantially in the lungs of pigs reaching virus titers up to 10^4 EID_{50}/ml at day 3 p.i. At day 6, the presence of the virus in one infected animal was still detectable. All animals cleared the infections by day 21 since all samples became negative in both virological and molecular tests. The presence of the virus in the BALFs was also checked in embryonated eggs and by qRT-PCR. A good correlation was observed between the virus titers in lungs and the qRT-PCR results for BALF samples (Table 6). On the whole, these results indicated that the selected H3N8 viruses from a wild aquatic reservoir can infect and cause lesions in the respiratory tracts of pigs without previous adaptation.

In addition, nasal swabs from infected animals were taken every other day for 10 days in a row to assess viral shedding. Viral shedding was only detected in the Sw IAV-infected group, but it was not detected in the rest of pigs. In fact, at day 2 p.i., only 3 of 12 animals shed virus, and at day 4 p.i. only 4 of 8 animals shed virus (data not shown).

Pigs infected with H3N8 avian IAVs had undetectable antibodies in the HAI test. All pigs at the beginning of this experiment were seronegative for IAV, as determined by a IAV blocking ELISA against NP (data not shown). All infected animals except the mock-infected group seroconverted against NP protein by day 21 p.i. (Table 7). Even pigs infected with the Eq and Ca IAVs that did not have a detectable infection showed antibodies against NP.

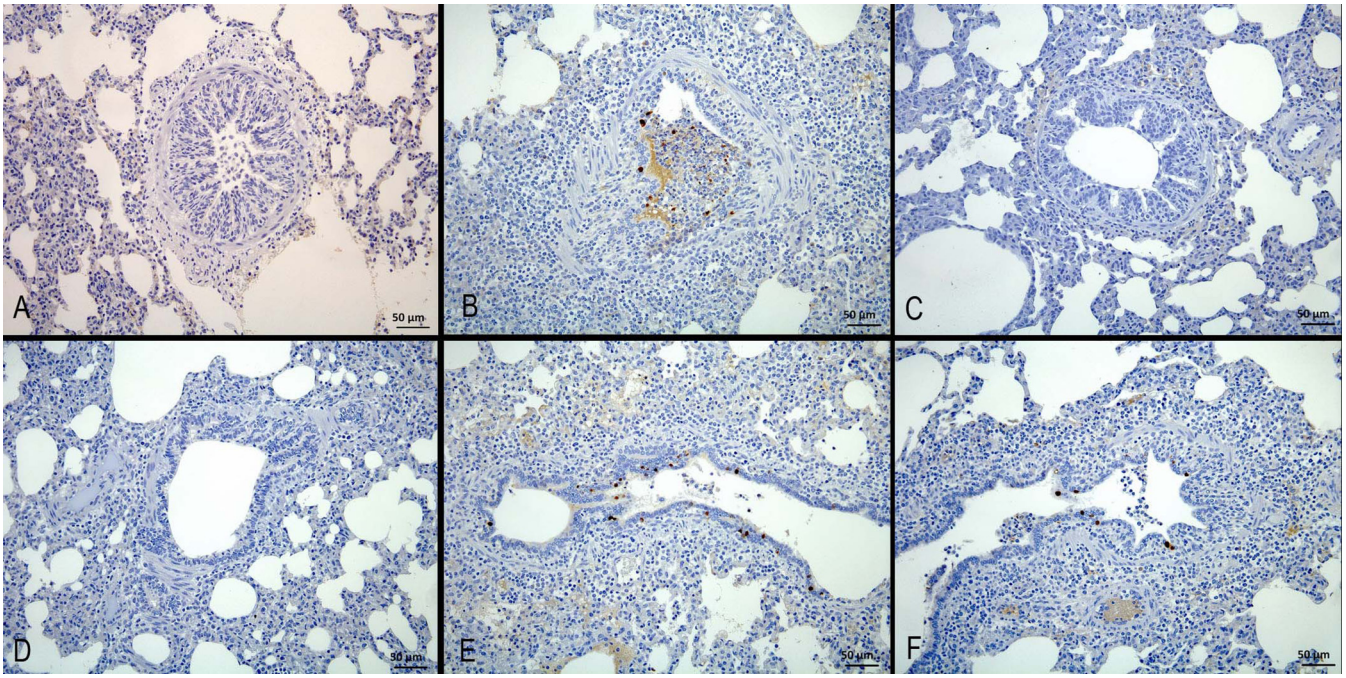


FIG 4 Immunohistochemical detection of IAV antigen at 3 dpi in the lungs of pigs infected with different IAVs. (A) Negative control. (B) Swine (Sw) IAV. There is positive nuclear staining in a few epithelial and desquamated cells of the bronchioli. (C) Equine (Eq) IAV. Note the negative staining. (D) Canine (Ca) IAV. Note the negative staining. (E) Avian (Av) IAV. There is positive nuclear staining in a few epithelial cells of the bronchioli. (F) Seal (Se) IAV. There is positive nuclear staining in a few epithelial cells of the bronchioli.

TABLE 6 Virus titers obtained by egg inoculation and real-time PCR results for lungs and bronchoalveolar lavage fluids collected from infected pigs[a]

Virus	Virus titer (EID_{50}/ml) in lung			RT-PCR (C_T) result in BALF		
	Day 3	Day 6	Day 21	Day 3	Day 6	Day 21
Mock-1	0/0	0/0	0/0	0	0	0
Mock-2	0/0	0/0	0/0	0	0	0
Sw-1	$10^{>7}$/18	10^2/25	0/0	26	32	0
Sw-2	(5.6×10^6)/19	10^2/27	0/0	25	26	0
Sw-3	(5.6×10^6)/17	0/31	0/0	26	29	0
Sw-4	10^6/19	0/31	0/0	21	33	0
Eq-1	0/0	0/0	0/0	35	0	0
Eq-2	0/0	0/0	0/0	0	0	0
Eq-3	0/0	0/0	0/0	0	0	0
Eq-4	0/0	0/0	0/0	0	0	0
Ca-1	0/0	0/0	0/0	0	0	0
Ca-2	0/0	0/0	0/0	0	0	0
Ca-3	0/0	0/0	0/0	0	0	0
Ca-4	0/0	0/0	0/0	0	0	0
Av-1	(2.8×10^4)/28	0/0	0/0	0	0	0
Av-2	10/30	<10/30	10/0	0	0	0
Av-3	(4.5×10^3)/21	0/0	0/0	32	0	0
Av-4	(2.8×10^3)/32	0/0	0/0	0	0	0
Se-1	(2.8×10^2)/34	0/0	0/0	0	0	0
Se-2	(4.5×10^3)/31	<10/34	0/0	36	0	0
Se-3	<10/36	0/31	0/0	37	37	0
Se-4	(2.8×10^3)/34	0/0	0/0	33	0	0

[a] BALF, bronchoalveolar lavage fluid.

All of the Se IAV-infected animals and three of four in the Sw and Av IAV groups were positive for NP antibodies at day 6 pi. In the Eq and Ca IAV-infected groups, only one animal in each group was NP positive at this time point. All sera were tested for their ability to inhibit neuraminidase (NA) function as a measurement of the specific antibodies against NA. The data in Fig. 5 indicated that these antibodies were indeed present in pigs infected with Sw, Av, and Se viruses, whereas they were absent in the mock-infected pigs and the ones belonging to the Eq or the Ca virus groups. Finally, significant differences ($P < 0.05$) were observed in antibody titers against NA between the Sw/Av/Se viruses and the Eq/Ca/Mock groups. F5

Subsequently, antibodies against HA were measured using HAI, the reference assay for antibody detection for IAV infections (Table 7). Unexpectedly, we could only detect antibodies against its own HA (homologous HAI) at day 21 p.i. in the swine IAV-infected group. We could not detect antibodies in the other groups

TABLE 7 Serological results obtained by HI and NP ELISA

Virus	Titer at various times postinfection[a]				
		Day 6		Day 21	
	Day 3 (HI)	HI	NP ELISA	HI	NP ELISA
Mock-1	<20	<20	Neg	<20	Neg
Mock-2	<20	<20	Neg	<20	Neg
Sw-1	<20	<20	Pos	160	Pos
Sw-2	<20	<20	Neg	160	Pos
Sw-3	<20	<20	Pos	80	Pos
Sw-4	<20	40	Pos	40	Pos
Eq-1	<20	<20	NC	<20	Pos
Eq-2	<20	<20	Neg	<20	Pos
Eq-3	<20	<20	Neg	<20	Pos
Eq-4	<20	<20	Pos	<20	Pos
Ca-1	<20	<20	Neg	<20	Pos
Ca-2	<20	<20	Pos	<20	Pos
Ca-3	<20	<20	Neg	<20	Pos
Ca-4	<20	<20	Neg	<20	Pos
Av-1	<20	<20	Neg	<20	Pos
Av-2	<20	<20	Pos	<20	Pos
Av-3	<20	<20	Pos	<20	Pos
Av-4	<20	<20	Pos	<20	Pos
Se-1	<20	<20	Pos	<20	Pos
Se-2	<20	<20	Pos	<20	Pos
Se-3	<20	<20	Pos	<20	Pos
Se-4	<20	<20	Pos	<20	Pos

[a] The serological titer is expressed as the reciprocal of the serum dilution. Neg, negative; Pos, positive; NC, not conclusive.

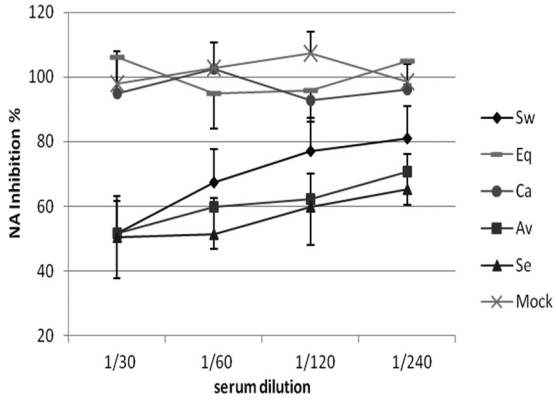


FIG 5 Neuraminidase (NA) inhibition by sera from pigs infected with each of the indicated IAVs. The data are means ± the standard deviations (SD) for four sera collected 21 days p.i. from animals inoculated with swine H3N2 (Sw), avian H3N8 (Av), seal H3N8 (Se), equine H3N8 (Eq), and canine H3N8 (Ca) IAVs and two sera from mock-infected pigs.

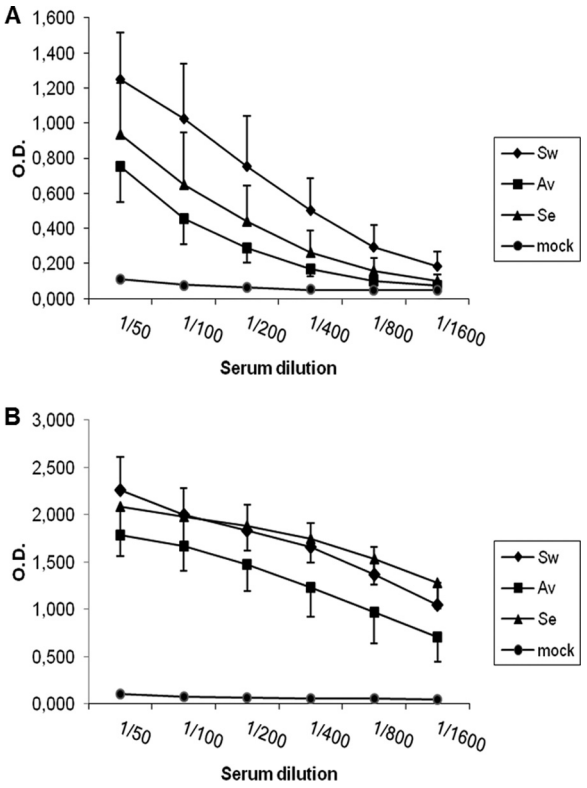


FIG 6 ELISAs were performed using purified A/PR/8/34 IAVs and sera of mice immunized intraperitoneally with 2.7×10^7 EID_{50} per mouse of swine H3N2 (Sw), avian H3N8 (Av), and seal H3N8 (Se) IAVs inactivated by UV. (A) ELISAs were performed using sera collected 9 days after the first immunization. (B) ELISA were performed using sera collected 14 days after the second immunization with the same amount of immunogen. The data are presented as the median ± the SD.

against the each virus even if the assay was repeated three times on three different days with differently treated samples. The titers ranged from 1:113 to 1:452 in the Sw IAV-infected pigs. Similar results were obtained in neutralization tests, since only sera from pigs infected with the swine-adapted virus strain showed the ability to neutralize the homologous virus (data not shown).

Sera from animals in different IAV-infected groups were also tested for cross-reactivity against different swine (H3N2 A/sw/It/312583/09, H1N1 A/sw/It/267505, and H1N2 A/sw/It/284922/09) and equine (H3N8 A/eq/New Market/93) IAVs. No cross-reactivity was detected by HAI testing in any sera, except for those coming from Sw IAV-infected animals, which showed HAI titers with an H3N2 subtype (data not showed).

Since failure to mount an HAI response was detected in NP-positive pigs infected with these viruses, the question was whether or not these results were peculiar to a particular animal species. Therefore, mice were intraperitoneally inoculated with the inactivated Sw, Av, and Se IAVs at a 2.8×10^7 EID_{50} were studied for antibody generation. All mice seroconverted when ELISA was used to determine the presence of antibodies against IAV in sera (Fig. 6A and B). However, Av and Se virus-inoculated mice did not show HAI antibodies. After the mice were boosted with a second dose, the Av virus-inoculated mice remained HAI negative, but some HAI titer seroconversion was observed in the Se virus-inoculated group (Table 8).

Analysis of the H3N8 avian IAV does not reveal mammalian adaptation signatures. The Sw H3N2, Eq H3N8, and Se H3N8 IAVs had been previously sequenced, and data were available at the NCBI database. We sequenced the Ca and Av IAV used for the experimental infection (accession numbers KP636491 to KP636498 and KP636475 to KP636482, respectively). Also, isolates from Sw, Av, and Se IAV strains detected in the lungs of infected pigs were sequenced. The Se IAV did not show any amino acid changes after replication in pigs, whereas Sw IAV detected in an infected pig showed an amino acid change D101N in the NP gene frequently observed in animal IAV (24). On the other hand, the Av H3N8 virus did not reveal mammalian adaptation signatures. However, the predicted amino acid sequence of HA showed a S110F mutation (H3 numbering), while the NA sequence showed a P152S mutation. The significance of these mutations requires further investigations since neither has been studied thoroughly. Phylogenetic trees of all eight segments confirmed the relationship between the avian and seal H3N8 viruses and the swine H3N2 virus (for seven segments, excluding the NA gene), which segregated separately from the equine and the canine viruses (Fig. 7).

DISCUSSION

The emergence and pandemic spread of the 2009 swine origin H1N1 IAV, despite the ongoing circulation of human H1N1 viruses, indicates that an antigenically distant animal origin, such as H3 virus, may pose a pandemic threat despite the circulation of H3N2 viruses in humans since 1968. On the other hand, even though direct transmission of Eq H3N8 IAV to humans has not been reported, infection of humans with Eq IAV under experimental conditions has been proven productive (4). The fact that Eq IAVs have the ability to infect humans and that these viruses

TABLE 8 Hemagglutination inhibition assay of sera from mice immunized intraperitoneally with swine H3N2 (Sw), avian H3N8 (Av), or seal H3N8 (Se) influenza viruses inactivated by UV irradiation[a]

	Mean titer ± SD					
	HI α-Sw-IV		HI α-Av-IV		HI α-Se-IV	
Serum	Preboost	Postboost	Preboost	Postboost	Preboost	Postboost
α-Sw	160 ± 0.0	806.3 ± 284.4	<20	<20	<20	<20
α-Av	<20	<20	<20	<20	<20	<20
α-Se	<20	<20	<20	<20	<20	53.3 ± 23.1
Mock	<20	<20	<20	<20	<20	<20

[a] Preboost, HI titers for sera collected 9 days after the first immunization; Postboost, HI titers for sera collected 14 days after the second immunization.

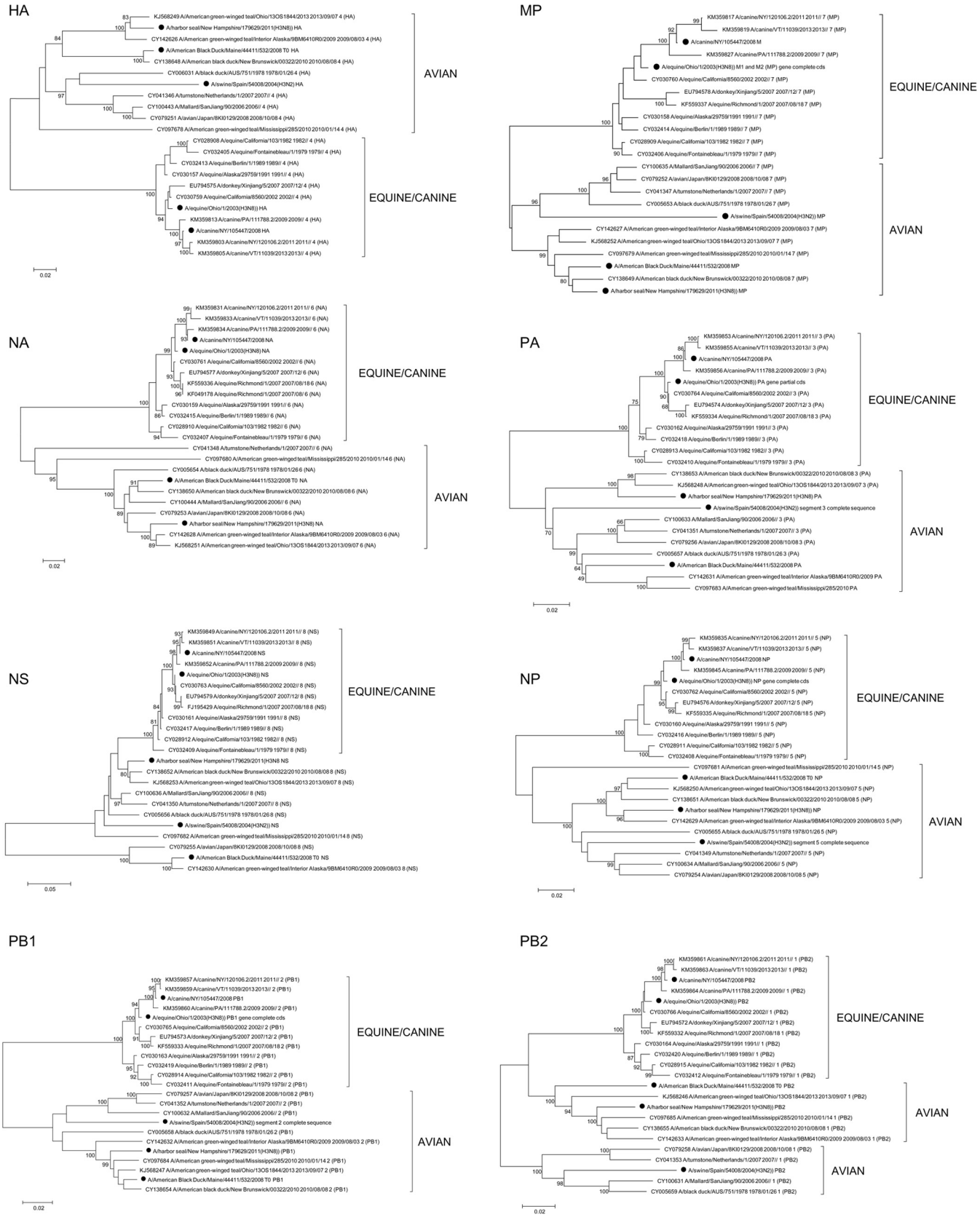


FIG 7 Neighbor-joining trees of eight gene segments of viruses examined in the present study (●) and H3N8 IAVs from equine (Eq), canine (Ca), and avian (Av) origins retrieved from GenBank.

have crossed the species barrier and infected dogs, pigs, and camels (25) emphasizes the potential threat posed to human health by viruses of this subtype. In addition, pigs may be easily exposed to equine or canine IAV from horses and dogs living in the same farm or after exposure to viruses from birds. Pigs were originally thought to be the mixing vessel where viruses from different origins could coinfect the host and new viruses with pandemic potential could arise (26).

The main aim of our study was to investigate the prospective ability of different H3N8 IAVs from different origins (Eq, Ca, Av, and Se) to productively infect pigs as a host for possible future reassortants with pandemic potential. A/Equine/1/OH/03 (H3N8) was chosen as the representative strain in North America for equine IAV belonging to clade I. This strain is currently the component of the equine influenza A virus vaccine for North America (2). Since the representative canine IAV for the canine influenza A virus vaccine is not available, a canine IAV strain was randomly selected to perform these experiments (A/Canine/NY/105447/08). A/American black duck/Maine/44411-174/2008 (H3N8) was chosen as an avian virus representative. The reason to choose this isolate was based on the experimental data on the binding ability of this virus to α-2,6 sialic acids (8). Finally, the seal virus A/Harbor Seal/New Hampshire/179629/2011 (H3N8) was included since it caused a fatal outbreak in seals and also for its ability to bind to mammalian IV receptors (8, 9). As positive control, we used a swine IAV belonging to the H3N2 subtype (A/Swine/Spain/54008/2004). This particular strain was isolated from an infected pig in a Spanish farm.

First, we evaluated the replication and binding abilities *in vitro* of an H3N8 set of viruses, and marked differences were observed among them. Subsequently, their hemagglutination abilities were studied to confirm sialic acid binding preferences by testing for their ability to bind to α-2,6 and α-2,3 linkages to galactose (receptors preferred by human and avian IAVs, respectively) in erythrocytes from several species preferentially expressing α-2,6 or α-2,3. On the whole, Eq and Ca viruses were the ones with the poorest ability to bind to pig erythrocytes (Table 2). Also, they were the ones with lower binding values to pig trachea explants and therefore the ones exhibiting the lowest values of viral replication (Tables 3 and 4). Recent results from Feng et al. (27) analyzing other related H3N8 canine and equine viruses indicated that canine and equine viruses show minimal biological differences despite phylogenetic divergence. In fact, both virus types bind more effectively to α-2,3-linked sialic acid than to α-2,6-linked sialic acid. These results are in agreement with the findings presented in here. Feng et al. showed that inoculation of both viruses into tracheal explants yielded similar levels of infection by each virus in dog tracheas, whereas equine viruses were more infectious than canine viruses in horse tracheas (27). However, in our experiments with pig trachea explants, the replication of Eq and Ca virus strains was minimal (Tables 3 and 4), indicating that factors other than sialic acid binding may play a role in infectivity and host susceptibility.

Altogether, our results indicate that Eq and Ca virus isolates may have little, if any, capacity to infect pigs, whereas Av and Se virus isolates retain this ability. This observation was based on evidence provided by Ito and coworkers showing that the porcine trachea contains sialic acids with α-2,6 and α-2,3 linkages to galactose (receptors preferred by human and avian IAVs, respectively). However, recent data from Van Poucke et al. showed that the sialic acid receptor distribution in pig respiratory tissue is similar to that in humans, showing abundant expression of α-2,6-linked sialic acid receptors in the trachea, as well as in other parts of the respiratory tract, whereas α-2,3-linked sialic acids were only detected in the bronchioles and alveoli (28, 29). The similarity in receptor specificity in humans has been clearly shown with the novel pandemic H1N1 virus from 2009, whereby a zoonotic event spread the virus through the human population and reverse zoonotic events caused pigs to be infected by human viruses.

In order to prove whether or not these sets of H3N8 virus were able to infect pigs, an experimental infection was performed. Infection took place intratracheally with 2×10^5 EID_{50} of each virus. This infectious dose was dictated by the poor growth of the Ca virus isolate in embryonated chicken eggs only. In accordance with our results *in vitro*, Eq and Ca viruses did not cause lesions, and they did not replicate in pig lungs, whereas the Av and Se strains did. Despite a low inoculation dose compared to other reported experimental infections (12, 28), the Av and Se IAVs were able to replicate substantially in the lungs of pigs, caused lesions, and maintained infection until day 6. However, no clinical respiratory symptoms were observed, and no virus shedding was detected in any H3N8 virus-infected pig (Fig. 2), indicating that horizontal transmission was unlikely to happen in a situation where these isolates infect pigs. Nevertheless, it cannot be ruled out that the low dose of inoculum and the administration route could affect the pattern of clinical outcome and shedding for H3N8 IAV.

Surprisingly, we could not detect specific antibodies against HA in any H3N8-infected pigs even though antibodies against NP were detected by ELISA. The fact that seroconversion against HA in the Eq and Ca IAV-infected pigs was not detected could be due to the absence of replication, but the fact that antibodies to HA were not detectable in the groups of pigs infected with H3N8 viruses with a bird origin (Av and Se) was an unpredicted event (Table 7). However, antibodies to NA were detected at 21 dpi only in pigs where replication was taking place, such with the Sw, Av, and Se virus isolates (Fig. 5). To determine whether this lack of antibody detection by HAI testing was due to a particular host, immunization experiments were conducted in mice. Similar results were obtained in mice and pigs, whereby HAI antibodies in Av and Se virus-infected animals were undetectable, whereas antibodies against IAV were detected (Table 8 and Fig. 6). These results indicate that the lack of HAI antibodies when pigs were infected with Av or Se viruses were not due to a particular host. A similar failure was also observed in experimental infections in pigs where various swine IAV subtypes were used for intratracheal inoculation (30). Therefore, special attention should focus on H3N8 subtype viruses, since they could behave as stealth viruses when HAI is used as a diagnostic tool in an important host such as swine, where further adaptation and the acquisition of human-like features could take place. Nevertheless, a possible caveat of the present study would be that only one H3N8 strain isolated from each host be used as a representative one. We cannot rule out that other H3N8 IAV strains could behave differently, and this is an area that merits further study.

Recently, a live attenuated, cold-adapted H3N8 virus has been engineered using reverse genetics (31), and the results suggest that a single dose of this H3N8 vaccine induced a protective response in mice against homologous and heterologous viruses, including the Se H3N8 virus used in our study. Serological data from human

and ferrets also suggest that this H3N8 vaccine may be protective. It remains to be explored whether this promising vaccine candidate would exhibit the same cross-reactivity and protective ability in pigs, given the ability of certain H3N8 viruses to infect pigs.

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We do not have any potential conflict of interest to declare.

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